Association of Interleukin-1B gene polymorphisms with Chronic and Aggressive forms of Periodontitis

A Senior Honors Thesis

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by

Sung Jin Min

The Ohio State University
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Project Advisor: Professor Donald Dean, Department of Biochemistry
Professor Pinar Emecen, Department of Periodontology
Professor John Walters, Department of Periodontology

Introduction:

Periodontitis is a disease of the periodontium characterized by inflammation of the gums and breakdown of the supporting structures of teeth. Periodontitis can be classified as aggressive or chronic periodontitis. Aggressive periodontitis is characterized by rapid attachment loss and bone destruction, and its patients are usually adolescents who are clinically healthy except for the presence of periodontitis (Lang et al, 1999). Chronic periodontitis is characterized by slow to moderate rate of progression and it is commonly detected in adults. Both disease entities can be further classified in terms of the number of sites involved (localized or generalized). If less than 30% of sites are affected, it is defined as localized. If more than 30% of sites are affected, it is defined as generalized. (Lindhe et al, 1999).

The initiation of periodontitis occurs when predominantly gram-negative, anaerobic or microaerophilic bacteria colonize the subgingival area. (Page, 1997) At the 1996 World Workshop on Clinical Periodontics, the relevant working group concluded that most human periodontitis is caused by *Porphyromonas gingivalis, Bacteroids forsythus* and *Actinobacillus actinomycetemcomitans*. While the etiology of periodontitis is bacterial, the pathogenesis of disease is mediated by the host response. In general, the colonization of bacteria stimulates the host defense mechanism against infection, which produces a variety of host molecules to neutralize the bacterial challenge. (Kornman, 1997) However, the host system, which is normally protective, causes tissue damage when the balance between the host system and bacteria is disrupted. (Page, 1997)

The balance between host defense mechanisms and bacteria can be disrupted when periodontal bacteria overcome the protective mechanism of human body, mainly provided by epithelial barriers, antibodies, complement proteins and neutrophils (Hart et al, 1994; Miyasaki et al, 1991). There are several ways for periodontal bacteria to overcome these

protective host mechanisms. First of all, some bacteria, such as *A. actinomycetemcomitans* or *Campylobacter rectus*, produce leukotoxins that kill neutrophils directly (Tsai et al, 1979;Gillespie, Smutko et al,1993). Secondly, some bacteria, such as *P. gingivalis,* produce proteolytic enzymes that reduce the level of antibody and complement proteins (Cutler, Arnold, Schenkein et al, 1993; Schenkein et al, 1989). Third, some bacteria such as *A. actinomycetemcomitans* produce factors that suppress the immune response (Shenker, Vitale, Welham et al, 1990; Shenker, Tsai, Taichman et al, 1982). Finally, some bacteria invade tissue cells so that they can avoid contact with neutrophils and molecules of immune system (Fives-Taylor, Meyer, Mintz et al, 1995; Lamont, Chan, Belton, Izutsu et al, 1995; Holt and Brammanti, 1991). Increased and persistent levels of microorganisms and the consequent production of its metabolic by-products affect the gingival vasculature and provide the environment and nutrients that allow putative pathogens to flourish. (Moore and Moore. 1994) As a result, more virulent microorganisms become concentrated within the plaque mass.

Once the major protective elements in the periodontium have been overwhelmed by bacteria, a number of host-mediated destructive processes are also initiated. First of all, neutrophil-mediated tissue injury occurs. Polymorphonuclear leukocytes, which normally provide protection, contribute to tissue damage by spilling some of proteolytic enzymes during degranulation process, which, in turn, degrade surrounding host cells (Van Dyke, 2003). Moreover, bacterial constituents such as lipopolysaccharide (LPS) engages with monocytes, lymphocytes, fibroblast, and other host cells and stimulate production of both catabolic cytokines and inflammatory mediators including arachidonic acid metabolites such as prostaglandin E_2 (PGE_2). Such cytokines and inflammatory mediators in turn cause the release of tissue-derived enzymes, the matrix metalloproteinases and the mobilization of the host tissues, and results in establishment of tissue destruction in periodontitis lesions

(Offenbacher, 1996; Birkedal-Hansen, 1993). In this process, cytokines are crucial in molecular signaling, and are responsible for inflammatory changes related with periodontal disease by affecting the metabolism of many other surrounding tissues.

Among cytokines released from surrounding host cells, interleukin I (IL-1) has some of the most significant effects. IL-1 is a very potent multifunction cytokine that appears to be a central regulator of the inflammatory and immune responses. It has numerous effects on bone. It increases inflammatory cell recruitment and inhibits bone formation, by binding to osteoblast cells. The effects of IL-1 on ostetoblasts include stimulation of prostaglandin formation and modulation of gene expression of several proteins, such as cytokines, enzymes, enzyme inhibitors, matrix proteins, and so forth. (Tatakis, 1993) This suggests that IL-1 may be involved in the pathogenesis of bone disease, and previous studies of periodontitis using IL-1 and Tumor Necrosis Factor (TNF) antagonists confirms that significant component of the pathologic process of periodontitis is due to IL-1/TNF activity. (Graves et al., 1998)

The production and activity of IL-1 is regulated by both transcriptional and posttranslational mechanisms, as well as by the IL-1 receptor antagonist (IL-1ra). IL-1ra counterbalances the potentially injurious proinflammatory effects of IL-1β. Functional polymorphisms in IL-1B promoter region could impair the interplay between IL-1 and IL-1ra, causing an unfavorable high IL-1/IL-1ra ratio. Several polymorphisms were identified in the IL-1B region, including -511 and -31 in the promoter region, and +3954 in exon 5. (Bioque et al, 1995; Di Giovine et al, 1992; Pociot et al, 1992) It has been indicated that -511 and -31 polymorphisms inducing unfavorable IL-1/IL-1ra ratio influences higher susceptibility to and prognosis of diseases possibly due to IL-1β-mediated inflammation. (Moos et al, 2000; El-Omar et al, 2000; Bioque et al, 1995) In terms of periodontitis occurrence, a previous study has shown the association of single nuclear polymorphisms at IL-1B-511 with periodontitis occurrence, while IL-1B-3954 was not significantly associated.

(Emecen et al, under review) However, previous studies imply that the effects of the promoter SNPs on gene transcription and regulation could involve many interactions among multiple polymorphic sites as a complex. (Terry et al., 2000; Drysdale et al., 2000; Daly et al., 2001) Therefore, we have used a case-control approach to study the association of haplotypes in the region of the interleukin 1 gene with generalized aggressive periodontitis and generalized chronic periodontitis in a Turkish population.

Materials and Methods:

Study subjects

A cross-sectional study design was used with patients who registered for treatment at Hacettepe University, Faculty of Dentistry, Department of Periodontology. The study protocol was approved by the Ethics Committee of Hacettepe University Medical Sciences Review Board and the Ohio State University Biomedical Institutional Review Board. Informed consent was obtained from all participants in accordance with the Helsinki Declaration.

The study population included three groups; generalized aggressive periodontitis (GAP), generalized chronic periodontitis (GCP) patients and periodontally healthy controls (HC) diagnosed by criteria based on AAP's 1999 International Workshop for a Classification of Periodontal Diseases and Conditions. In the present study, 52 generalized aggressive patients, 53 generalized chronic patients, and 42 periodontally healthy controls were evaluated for 3 IL-1B gene polymorphisms. Two of these, IL-1B -31 and IL-1B -511, are located on the promoter region, and one, IL-1B +3954, on the 5^{th} exon.

All participants were non-smokers and had at least 20 teeth in the mouth. None of them had a serious systemic disease impairing the immune system. Exclusion criteria also included medical disorders like diabetes that will affect periodontal pathogenesis, pregnancy and lactating. Diagnoses were confirmed by periapical or panoramic radiographs and periodontal examination performed by a single examiner. The clinical measurements included measurement of probing depth (PD), clinical attachment loss (CAL), plaque index (PI) and bleeding on probing (BOP). Probing depth and CAL were measured at six locations around each tooth and median measurement was calculated and used for comparisons. Plaque index was recorded according to Sillness and Loe (1964), while BOP was recorded as percentage of bleeding sites compared total available tooth surfaces.

Isolation of genomic DNA

Genomic DNA was extracted from peripheral blood samples by using a salting out procedure (Miller, 1986). The PCR amplifications were performed in 50 μl volume. The primer selection and PCR amplification were carried out according to Kornman et al. (1997). DNA (100-500 ng) was mixed with 48 μl of Supermix, 1 μl of forward and reverse primer, 1 μl of distilled water. The enzyme digestion was carried out by mixing 17 μl of PCR product with 10U of corresponding restriction enzyme and incubating for 16 hours at 37°C.

***IL-1B-31*:** The SNP at position 31 of IL-1B gene was amplified using the thermocycling conditions of denaturation for 2 min at 96 °C followed by 35 cycles of denaturation for 1 min at 94 °C, annealing of primers for 1 min at 50 °C, primer extension for 1 min at 72 °C and final extension for 2 min at 72 °C. The PCR products were digested with 10U of *Alu I* endonuclease at 37 °C for 16 hours. The resulting PCR products 83bp+150bp (allele 1,T) and 234bp (allele 2, C) were visualized on 3% agarose gel electrophoresis as 125V for 45 min, which contains ethidium bromide for staining. The heterozygous individuals were identified by the presence of two bands.

IL-1B-511: The SNP at position -511 of IL-1B gene was amplified using the thermocycling conditions of denaturation for 2 min at 95 °C followed by 35 cycles of denaturation for 1 min at 94 °C, annealing of primers for 1 min at 53 °C, primer extension for 1 min at 74 °C and final extension for 5 min at 72 °C. The PCR products were digested with 10U of *Ava I* endonuclease at 37 °C for overnight. The resulting PCR products 190bp+114bp (allele 1, C) and 304bp (allele 2, T) were visualized on 3% agarose gel electrophoresis as 130V for 30 min and ethidium bromide staining. The heterozygous individuals were identified by the presence of three bands.

IL-1B +3954: The SNP at position +3954 of IL-1B gene was amplified using the thermocycling conditions of denaturation for 2 min at 95 °C followed by 35 cycles of denaturation for 1 min at 95 °C, annealing of primers for 1 min at 67.5 °C, primer extension for 1 min at 74 °C and final extension for 2 min at 72 °C. The PCR products were digested with 10U of *Taq I* endonuclease at 65 °C for overnight. The resulting PCR products 97bp+85bp+12bp (allele 1, C) and 184bp+12bp (allele 2, T) were visualized on 4% agarose gel electrophoresis as 130V for 30 min and ethidium bromide staining. The heterozygous individuals were identified by the presence of three bands.

Data Analysis:

The allele association and allele frequencies in GAP, GCP and HC groups were analyzed by using the Chi-Square test. The different haplotypes and distribution of haplotype frequencies between groups were determined using HelixTree software trial package. This program uses the Expectation-Maximization (EM) algorithm and the Composite Haplotype Method algorithm to calculate haplotype frequencies with 95% confidence interval. The EM algorithm provides the maximum likelihood estimates of the frequencies of haplotypes (Excoffier and Slatkin, 1995).

Results:

Allele frequencies of IL-1β SNPs in GAP case, GCP case and control groups

IL-1β polymorphisms were detected at positions -31, -511, and +3954 in the GAP case, GCP case and healthy control groups. The allele frequencies for these three SNPs in the GAP case and GCP case groups were not significantly different from those observed in the control group (Table 2, Table 3). There was an over-representation of -31C and -511T in the GAP case group relative to the control group, but the differences between case and control groups were not statistically significant (P = 0.13 and P=0.063, respectively).

Analysis of allele association

The significance of the association between each of the three IL-1β SNPs and case/control status was examined. The -31T>C and -511C>T SNPs exhibited a significant association with GAP cases (P = 0.024 and P = 0.017, respectively), but there was no apparent association between +3954C>T and GAP (Table 4). None of these SNPs exhibited a significant association with GCP (Table 5).

Haplotype analysis

Haplotype frequency analysis delineated seven haplotypes for subjects in the GAP group, six haplotypes in the GCP group and five haplotypes in the control group (Table 6). The four most common haplotypes found in all groups were -31C/-511T/+3954C, -31T/-511C/+3954C, -31T/,-511C/+3954T and -31C/-511T/+3954T. The most common haplotype in the GCP group and the control group was -31T/-511C/+3954C, while the most common haplotype in the GAP group was -31C/-511T/+3954C.

With respect to -31C/-511T/+3954C and -31C/-511T/+3954T haplotypes, the frequencies observed in the GAP group were higher than those observed in the GCP or

control groups. However, with respect to -31T/-511C/+3954C and -31T/-511C/+3954T haplotypes, the frequencies observed in the GAP group were lower than those observed in the GCP or control groups.

In both GAP and GCP groups, no haplotypes had significantly different frequency from the control group when comparing 95% confidence interval range of EM frequencies (Figure 1). All comparisons showed the overlap of 95% confidence interval range of EM frequencies between the case and the control group.

Discussion:

Since Kornman et al. (1997) reported an association between IL-1 polymorphisms in the promoter region and adult periodontitis, several other studies have attempted to confirm this relationship. Many of these studies concentrated on the single nucleotide polymorphisms IL-1A +4845 and IL-1B +3954 on chromosome 2q.13. However, the results from several studies were not consistent, and it has not been possible to find the truly causative agent of periodontitis from the individual single nucleotide polymorphism approach. For example, Parkhill et al. (2000) and Diehl et al. (1999) reported that the homozygotes of allele 1 of IL-1B +3954 had an increased risk of developing aggressive periodontitis, while Lopez et al. (2005) found that the carriage of homozygous for allele 1 of IL-1B +3954 appeared to be a protective factor for periodontitis, and the heterozygous genotype of the IL-1B +3954 showed a strong positive association with periodontitis. According to Knight (2005), the single nucleotide polymorphism approach is likely to mislead unless the SNP itself can be a sufficient etiological event or a reliable marker of a disease-related haplotype.

Since an understanding of haplotype structure across segments of chromosomes that contain potential disease genes is likely to increase the value of genetic association (Horne et al, 2004; Crawford et al, 2005; Tabor et al, 2002), in the present studies, we chose two other SNPs to investigate among SNPs identified in the IL1B gene by Chen et al. (2006), along with IL-1B +3954 SNP.. Previous haplotype analysis papers in IL-1B gene were reviewed (Wen et al, 2006; Hall et al, 2004; El-Omar et al, 2000; Chen et al, 2006), and SNPs at IL-1B -31 and -511 were chosen to be analyzed together with IL-1B +3954 SNP for the IL-1B promoter function of individual SNPs alone and within the haplotype sequences.

In a single SNP analysis, IL-1B -31T>C SNPs was associated with GAP (P=0.024). This is the first report on the association between IL-1B -31 SNP and periodontitis risk. In the GAP group, subjects who had IL-1B -31T/T genotype exhibited lower frequencies than

the control group, and subjects who had IL-1B -31T/C genotype exhibited higher frequencies than the control group. No significant difference was found from the distribution of allele frequencies at IL-1B -31.

At position IL-1B -511, its genotype also exhibited a significant association with GAP (P=0.017). The homozygous pair of C allele decreased, and the heterozygous pair of T allele and C allele increased significantly in GAP group, which is consistant with the male GAP group of Chinese population from Li et al. (2004). Li et al. (2004) reported that the IL-1B -511 T/C heterozygote frequency was significantly increased in the male GAP group compared to male controls with marginal evidence for association (P=0.048), while there was no statistically significant difference in the distribution of the genotypes and alleles at IL-1B -511 between GAP and control groups. No significant difference was found from the distribution of allele frequencies at IL-1B -511.

There were no statistically significant differences in the distributions of the genotypes and alleles for IL-1B +3954 among three groups, which is contradict with many of previous papers (Parkhill et al, 2000; Diehl et al, 1999; Lopez et al, 2005; Rogers et al, 2002; Quappe et al, 2004).

In our haplotype analysis, no significant differences between case and control groups were detected with respect to the predominant haplotypes. However, we detected the increase of the frequency from two haplotypes which contain rare alleles at -31 and -511, -31C/-511T/+3954C and -31C/-511T/+3954T, and the decrease of the frequency from two haplotypes containing common alleles at -31 and -511, -31T/-511C/+3954C and -31T/-511C/+3954T. One possible explanation of this trait is that IL-1B-31 and IL-1B -511 are in essentially complete linkage disequilibrium in all populations of which we are aware (El-Omar et al, 2000; Lee et al, 2004; Muraki Y et al, 2004; Hall et al, 2004). This combination may be an example of two SNPs that evolved together as a result of biological activity based

on a *cis*-interaction (Guasch et al. 1996). This linkage disequilibrium can be related with the allele frequency analysis from our study. Both the IL-1B -31C allele and the IL-1B -511T allele was overrepresented in the GAP group relative to the control group although this was not statistically significant (P=0.13 and P=0.063 respectively).

In ex vivo studies of LPS-induced IL-1β secretion, Hall et al. (2004) reported that the IL-1B haplotype, composed of -31C and -511T allele, was significantly associated with a 2-3 fold increase in LPS-induced IL-1β protein secretion. In addition, it seems to be associated with enhanced release of newly synthesized IL-1β protein, which is consistent with our findings. However, Wen et al. (2006) reported that IL-1β level was higher in homozygotes of the -31T-511C haplotype from the analysis with use of the haplotype containing only the -31 and -511 polymorphisms, which contradicts the report from Hall et al. (2004). One possible reason for the disagreement is the difference of the population. Hall et al. (2004) examined 31 healthy controls and 24 white subjects with rheumatoid arthritis, while Wen et al. (2006) examined 105 healthy subjects only. Ex vivo studies with periodontitis subjects and healthy controls is a reasonable approach for the further study for correlation with case-control studies.

In conclusion, polymorphisms in IL-1B -31 and -511 may contribute to the risk of developing GAP, while IL-1B +3954 polymorphisms do not appeared to be associated with an increased risk of developing periodontitis in a Turkish population. From the haplotype analysis, none of detected haplotypes was significantly associated with periodontitis risk, even though haplotypes with -31C and -511T exhibited higher frequencies in the GAP group. Further haplotype analysis with other SNPs in IL-1B is recommended due to the possibility that the causative agents throughout the IL-1 cluster on the 2q.13 region could be in linkage disequilibrium with -31C and -511T.

Table 1. Primers for Analysis of IL-1B polymorphisms

Polymorphisms	**Primers**
IL-1B -31	Forward-TCTTTTCCCCTTTCCTTTAACT
	Reverse-GAGAGACTCCCTTAGCACCTAGT
IL-1B -511	Forward-TGGCATTGATCTGGTTCATC
	Reverse-GTTTAGGAATCTTCCCACTT
IL-1B +3954	Forward-CTCAGGTGTCCTCGAAGAAATCAAA
	Reverse-GCTTTTTTGCTGTGAGTCCCG

Table 2. Allele frequencies of IL-1B SNPs in generalized aggressive periodontitis (GAP) and control groups

SNP		GAP n (freq)	Control n (freq)	P value*
IL-1ß -31	T	52 (0.50)	51 (0.62)	0.130
	C	52 (0.50)	31 (0.38)	
IL-1ß -511	C	48 (0.46)	50 (0.61)	0.063
	T	56 (0.54)	32 (0.39)	
IL-1ß +3954	C	79 (0.76)	63 (0.77)	0.972
	T	25 (0.24)	19 (0.23)	

* Chi-Square test

Table 3. Allele frequencies of IL-1B SNPs in generalized chronic periodontitis (GCP) and control groups

SNP		GCP n (freq)	Control n (freq)	P value*
IL-1ß -31	T	70 (0.66)	51 (0.62)	0.695
	C	36 (0.34)	31 (0.38)	
IL-1ß -511	C	71 (0.67)	50 (0.61)	0.484
	T	35 (0.33)	32 (0.39)	
IL-1ß +3954	C	74 (0.70)	63 (0.77)	0.364
	T	32 (0.30)	19 (0.23)	

* Chi-Square test

Table 4. Allele association of IL-1B SNPs with generalized aggressive periodontitis (GAP)

SNP		GAP n (freq)	Control n (freq)	P value*
IL-1ß -31	TT	10 (0.19)	18 (0.44)	0.024
	TC	32 (0.62)	15 (0.37)	
	CC	10 (0.19)	8 (0.19)	
IL-1ß -511	CC	8 (0.15)	17 (0.41)	0.017
	TC	32 (0.62)	16 (0.39)	
	TT	12 (0.23)	8 (0.20)	
IL-1ß +3954	CC	30 (0.58)	26 (0.63)	0.528
	TC	19 (0.36)	11 (0.27)	
	TT	3 (0.06)	4 (0.10)	

* Chi-Square test

Table 5. Allele association of IL-1B SNPs with generalized chronic periodontitis (GCP)

SNP		GCP n (freq)	Control n (freq)	P value
IL-1ß -31	TT	23 (0.43)	18 (0.44)	0.481
	TC	24 (0.46)	15 (0.37)	
	CC	6 (0.11)	8 (0.19)	
IL-1ß -511	CC	23 (0.44)	17 (0.41)	0.355
	TC	25 (0.47)	16 (0.39)	
	TT	5 (0.09)	8 (0.20)	
IL-1ß +3954	CC	27 (0.51)	26 (0.63)	0.467
	TC	20 (0.38)	11 (0.27)	
	TT	6 (0.11)	4 (0.10)	

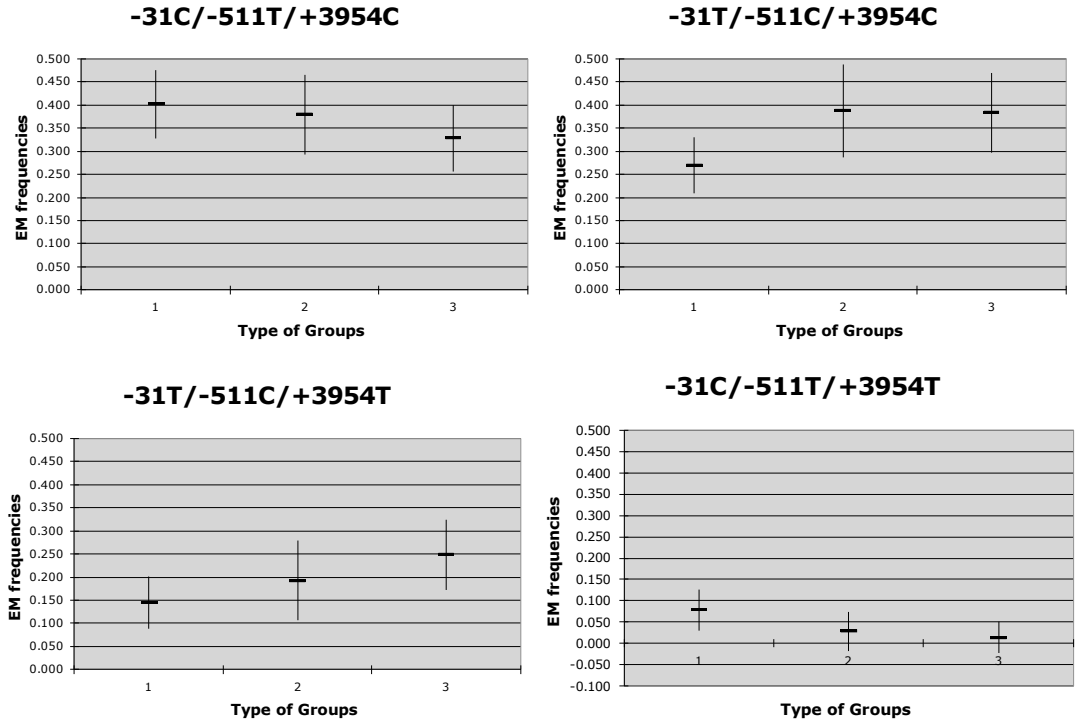
* Chi-Square test

Table 6. IL-1B haplotype frequencies in generalized aggressive periodontitis, generalized chronic periodontitis and control groups

Haplotype IL-1B (-31, -511, +3954)	GAP		Control		GCP	
	Mean	C.I.	Mean	C.I.	Mean	C.I.
C,T,C	0.401	0.073	0.379	0.085	0.328	0.071
T,C,C	0.269	0.061	0.387	0.100	0.383	0.085
T,C,T	0.144	0.057	0.193	0.086	0.248	0.076
C,T,T	0.078	0.048	0.028	0.046	0.014	0.036
T,T,C	0.077	0.059	0.013	0.038	nd	nd
C,C,C	0.025	0.032	nd	nd	nd	nd
C,C,T	0.005	0.027	nd	nd	0.018	0.032
T,T,T	Nd	nd	nd	nd	0.009	0.028

* The Expectation-Maximization algorithm was used to calculate mean value and confidence interval.

Figure 1. Range of EM frequencies of common haplotypes of groups in 95% confidence interval



* Group 1 is GAP group, Group 2 is control group, and Group 3 is GCP group. The mean value of EM frequencies is drawn as horizontal block, and 95% confidence interval of EM frequencies is drawn as a vertical line.

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